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A New Mutation in *mgrB* Mediating Polymyxin Resistance in *Klebsiella variicola*

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Short running title: Polymyxin-resistant *Klebsiella variicola*.

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Abstract

Polymyxin resistance is a public health concern, present in humans, animals and the environment, occasioned by chromosomal- or plasmid-encoding mechanisms. Chromosomal alterations in MgrB are frequently detected in *Klebsiella* spp., but not yet reported and characterised in *K. variicola*. Here, we performed microbiological and genomic characterisation of three polymyxin-resistant *K. variicola* isolates (M14, M15, and M50) recovered from the microbiota of migratory birds in Brazil. The isolates were submitted to SpeI-PFGE, broth microdilution, and Whole Genome Sequencing using Illumina MiSeq for analysis of genetic relatedness, sequence typing, and detection of antimicrobial resistance genes. *K. variicola* isolates belonged to two clones, and susceptibility tests showed resistance only for polymyxins. Sequences of chromosomal two-component systems (PmrAB, PhoPQ, RstAB, CrrAB) and MgrB were evaluated by blastN and blastP, against a polymyxin-susceptible *K. variicola* (A58243), and mutations biological effect was checked by the PROVEAN tool. In M14 and M15, *phoQ* mutations (D90N, I122S, and G385S) were identified, while in M50 a *mgrB* variant containing a single deletion (C deletion on position 93) leading to the production of a non-functional protein was detected. *mgrB* complementation studies showed restoration of polymyxin susceptibility (64 to ≤0.25 mg/L) when a WT *mgrB* was inserted into the *mgrB* deficient M50. This data confirmed the role of a non-functional *mgrB* variant in conferring polymyxin resistance, stressing the role of this regulator in *K. variicola* and drawing attention to novel polymyxin resistance mechanisms emerging in wildlife.

Keywords: Enterobacterales, Two-component systems, migratory birds, Antimicrobial Resistance

1. Introduction

Polymyxins are polypeptide antibiotics broadly used in human and veterinary medicine due to their great activity against Gran-negative bacteria, mainly against those pathogens with a multidrug-resistant (MDR) profile [1]. There have been reports of polymyxin-resistant *Enterobacterales* recovered from distinct settings in recent years, either by chromosomal or plasmid-mediated mechanisms [1]. Plasmid-mediated mechanisms, represented by mobile colistin resistance (*mcr*) genes, are often reported in Enterobacterales; however, they are more prevalent in food-animal samples than in humans and food products [2].

Chromosomal mechanisms leading to polymyxin resistance are closely associated with lipid A modification after a series of genetic and biochemical events coordinated by Two-Component Systems (TCS) such as PmrAB, PhoPQ, RstAB and CrrAB [3,4]. In 2014, the role of MgrB, a small protein responsible for negatively regulating PhoPQ activity, was described as the main mechanism driven polymyxin resistance in *Klebsiella pneumoniae* [5]. Since then, this mechanism has been worldwide reported [6-8] in *K. pneumoniae* and *K. oxytoca* [1,9], and more recently in *Enterobacter* spp. [10]. However, to date, no data associating polymyxin resistance to *mgrB* disruptions in *K. variicola* has been published. Herein, we have characterised three polymyxin-resistant *K. variicola* isolates recovered from the microbiota of migratory birds, ultimately mediated by chromosomal mutations.

2. Material and Methods

2.1. Bacterial strains. A surveillance study in 2012 aimed to identify antimicrobial-resistant bacteria carried by migratory and zoo resident birds in São Paulo, Brazil [11]. For this purpose, birds' choanal and cloacal swabs were collected and further plated on agar plates containing antimicrobial agents to favor the selection of resistant microorganisms. *K. variicola* isolates were recovered from

the choana of distinct *Dendrocygna viduata* birds following cultivation onto MacConkey agar containing 2 mg/L of polymyxin and further characterized in this study. The recovered isolates were identified by MALDI-TOF MS (Bruker Daltonics, Massachusetts, EUA) and 16S rRNA DNA-sequencing.

2.2. Antimicrobial susceptibility testing and molecular typing. Susceptibility profiles were determined by broth microdilution and interpreted according to EUCAST guidelines [12]. In addition, to check for the presence of polymyxin resistance mechanism-dependent of divalent ions such as MCR-like proteins, polymyxin B MICs were also determined in the presence of 10 mM of EDTA. Genetic relatedness was established by SpeI pulsed-field gel electrophoresis (PFGE) and interpreted using Tenover criteria [13].

2.3. Whole Genome Sequence Analysis. For genomic purposes, the isolates were whole genome sequenced using Illumina MiSeq (NexteraXTv2 and MiSeqReagent V3 Kit; 2×300cycles). Besides, a polymyxin-susceptible *K. variicola* strain (A58243) recovered from human-infection was also sequenced and used as a control. Raw sequences (fastq) were trimmed using Trim Galore (v0.5.0) and assembled into contigs using SPAdes (v3.9.0) [14]. The genomes were submitted to RAST (http://rast.nmpdr.org/) for automatic annotation followed by analysis on ResFinder (https://cge.cbs.dtu.dk/services/ResFinder-3.0/), PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder-2.0/), and *K. variicola* MLST website [15]. Mutation analysis of TCS (PmrAB, PmrD, PhoPQ, RstAB, CpxAR) and *mgrB* were conducted using as reference four genomes of *K. variicola* described as polymyxin-susceptible on NCBI (CP028555.1, CP016344.1, CP017289.1, and NZ_CEGG01000025.1) and the strain *K. variicola* A58243

(polymyxin MIC 0.25 μg/mL). Mutation impact in proteins was determined by PROVEAN (http://provean.jcvi.org/index.php), using threshold values bellow to -2.5 as biologically significant.

2.4. Expression level of TCS. To ascertain the expression levels of *pmrA, pmrB, phoP, phoQ* and *mgrB* we performed qRT-PCR. Briefly, total RNA was isolated from *K. variicola* isolates using RNeasy Mini Kit (Qiagen, Hilden, Germany) with addition of RNase-free DNase (Qiagen, Hilden, Germany). Reverse transcription of the extracted RNA was performed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), followed by transcripts quantification, performed in triplicate using SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and the 7500 Real Time (Life Technologies, Carlsbad, CA, USA). For this purpose, 16S rRNA was used as endogenous control, and the levels of TCS and regulator gene expression were compared to *K. variicola* A58243.

2.5. *mgrB* complementation. To evaluate the relationship between this new *mgrB* mutation and polymyxin resistance in *K. variicola*, we performed the *mgrB* complementation on M50 isolate. Briefly, an apramycin resistance gene was amplified and cloned into pBluescript SK+ XceI site (Stratagene Inc.) to build pskA vector. The gene *mgr*B was amplified from *Klebsiella variicola* A58243 (*mgrB*-WT) and M50 (*mgrB*-mutant) using the primers *mgrB*-BamHI-ext-R 5'-CGGGATCCCGAAGGCGTTCATTCTACCACC-3' EcoRI-*mgrB*-var-F 5'-GGAATTCCTTAAGAAGGCCGTGTTATCC-3' and cloned into pskA. Clones were selected on LB agar plates supplemented with apramycin (50 mg/L) and the constructions verified by sanger sequencing (Fasteris, Geneva, Switzerland). Polymyxin B broth microdilution was performed to quantify any modification on the MIC after *mgrB* complementation.

3. Results and Discussion

Three *K. variicola* (M14, M15, and M50) were recovered from the choana of distinct *Dendrocygna viduata* birds following cultivation onto MacConkey agar containing polymyxin B. The three isolates were subject to SpeI-PFGE and this analysis suggested that the isolates M14 and M15 were genetically related and classified as subtypes of the pattern A, A1 and A2. In contrast, M50 isolate belonged to the pattern B (Table 1). Interestingly, the isolates were susceptible to all antimicrobial agents tested except for polymyxin B (Table 1).

Genomic and microbiological features of all isolates were displayed in table 1. Isolates M14 and M15 belonged to ST137, while the isolate M50 was assigned to a new sequencing type denominated ST167. According to MLST database, isolates belonging to ST137 were previously identified in Germany causing human infections, highlighting this bacteria species' ability to exert symbiotic and pathogenic roles (http://mlstkv.insp.mx/). No plasmids were detected in these isolates, and only chromosomal resistance genes were detected. The gene encoding the intrinsic β-lactamase LEN was detected, $bla_{\text{LEN-24}}$ in M14 and M15 isolates, and $bla_{\text{LEN-13}}$ in M50 isolate. LEN-enzymes are chromosomally encoded and show a high level of similarity to SHV β-lactamases, which are also chromosomally encoded by *K. pneumoniae* [16].

Mutations in PmrB, PhoQ, and RstB were identified in two isolates; however, only mutations in PhoQ were considered deleterious by PROVEAN (Table 1). The deleterious mutation D90N was detected in M14 and M15 isolates. Interestingly, genetic modifications in the same TCS leading to polymyxin resistance were described previously in a *K. variicola* isolate from China, albeit not at the same amino-acid position [17]. However, the D90N substitution has not been described as causing polymyxin resistance in *K. pneumoniae,* although this highly conserved amino acid among Enterobacterales has been proposed as crucial for PhoQ function [18]. While the PhoQ G385S substitution has been described (although not functionally demonstrated) in *K. pneumoniae*, the I122 has not been described so far and its role remains to be demonstrated [19].

The level of transcription of TCS corroborated with the data obtained from genomic analysis, with increased transcriptional levels to *pmrB*, *phoP,* and *phoQ* in the M14 isolate, while in the M15 isolate the transcriptional levels of *pmrB* and *phoQ* rose (Figure 1).

Although the isolate M50 did not show mutation on these TCS, a single nucleotide deletion (C deletion on position 93) was observed in *mgrB.* This deletion changed the amino acid residues sequence downstream of D31 (aspartic acid 31), thus encoding a non-functional MgrB protein of 52 amino acids versus 47 amino acids for the wild-type MgrB (Figure 1). qRT-PCR experiments showed that the transcription level of the mutated *mgrB* was almost 5 times higher (4.8 times) than the susceptible isolate (A58243) and 4.6 times higher than other polymyxin-resistant isolates studied without mutation in *mgrB* (Figure 1a). To the best of our knowledge, this is the first description of polymyxin resistance being mediated by deletion of a single nucleotide in *mgrB*, resulting in an unfunctional protein. To date, polymyxin resistance mediated by *mgrB* has been frequently associated with incorporation of insertion sequences within the gene or its promoter or point mutations generating premature stop codons [1,3].

The complementation of M50 by a WT *mgrB* resulted in a drop of polymyxin B MIC from 64 to ≤0.25 mg/L (Table 2), corroborating our initial suspicion on this mutation's role for conferring polymyxin resistance in *K. variicola*. The complementation with the deficient *mgrB* amplified from M50 isolate, did not show any impact in polymyxin B MIC, proving that this mutation caused the production of a non-functional MgrB protein (Table 2). Recently, six polymyxin-resistant *K. pneumoniae* strains were described carrying a duplication of 79 nucleotides in *mgrB,* resulting in an unfunctional MgrB, being 26 amino acids longer than expected [20]. These isolates were also polymyxin-resistant, and no other mechanism causing resistance to polymyxins was detected by WGS analysis [20]. This data further supports our findings since the production of an unfunctional MgrB protein is also detected in *K. pneumoniae*.

4. Conclusion

K. variicola is an emerging human pathogen that should be monitored, especially regarding antimicrobial resistance and virulence determinants. Our results show that although *K. variicola* isolates are likely to be very susceptible to many antimicrobial agents, the susceptibility to polymyxins cannot be indirectly predicted. The identification of polymyxin-resistant *K. variicola* in migratory birds, also reinforces the need of constant effort on One Health surveillance programs since the close relationship between human and animals can facilitate the spread of such resistance determinants to hospital settings.

Data availability

Whole genomic sequences of three studied polymyxin-resistant *K. variicola* isolates have been deposited in GenBank under accession numbers JAAGEJ000000000, JAAGEK000000000, JAAGEL000000000.

Conflict of interests

A.C.G. has recently received research funding and/or consultation fees from Cristália, Enthasis Therapeutics, InfectoPharm, Eurofarma, Pfizer, MSD, and Zambon. Other authors have nothing to declare.

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295 **Table 1.** Microbiologic and genetic features of *K. variicola* isolates evaluated in this study.

296

Microbiologial and Genetic Features	Bacteria Isolates			
	M14	M15	M50	A58243
Bacteria species identification by MALDI-TOF	*K. variicola*	*K. variicola*	*K. variicola*	*K. variicola*
Antimicrobial susceptibility testing[a]				
Colistin	128	64	64	0.25
Polymyxin B	128	128	64	0.25
Polymyxin B + EDTA (10 mM)	128	128	64	ND
Ceftazidime	0.25	0.25	0.25	≤0.25
Ceftriaxone	≤0.125	≤0.125	≤0.125	ND
Aztreonam	≤0.06	≤0.06	≤0.06	≤0.25
Levofloxacin	≤0.06	≤0.06	≤0.06	ND
Gentamicin	2	2	2	ND
Tobramicin	0.5	0.5	0.5	ND
Amikacin	2	2	2	2
Cefepime	≤0.125	≤0.125	≤0.125	≤0.25
Meropenem	≤0.06	≤0.06	≤0.06	≤0.06
Imipenem	≤0.125	≤0.125	≤0.125	0.25
Piperacillin-Tazobactam	2	2	4	8
Fosfomycin	16	16	4	8
Genomic Features				
PFGE	A1	A2	B1	ND

MLST	ST137	ST137	ST167	ND
Genome size (bp)	5,880,384	5,882,887	5,403,894	5,711,702
GC (%)	56.9	56.9	57.5	57.2
ORFs	5860	5855	5235	5566
RNAs	94	94	91	92
Contigs	105	104	77	83
Chromossomal beta-lactamase	*bla*$_{\text{LEN-24}}$	*bla*$_{\text{LEN-24}}$	*bla*$_{\text{LEN-13}}$	*bla*$_{\text{LEN-9}}$
Alteration analysis in TCS components and regulators				
PmrB	N13H E272K	N13H E272K	Not found	-
PhoQ	**D90N (deleterious)** D101N R116H	**D90N (deleterious)** D101N	Not found	-
RstB	**G385S (deleterious)** M82I	**I122S (deleterious)** M82I	Not found	-
MrgB	Not found	Not found	Deletion on 93 nct position	

[a]The antimicrobial susceptibility profile of *K. variicola* isolates was determined by agar dilution, except for polymyxin B, which was tested by broth microdilution according to the EUCAST/BrCAST guidelines. MICs were expressed in mg/L.

300 **Table 2.** Polymyxin B MICs of the laboratory derivative strains

Strains[a]	Mean Polymyxin B MIC (mg/L)[b]	Mean Colistin MIC (mg/L)[b]
M50	64	64
M50 + pskA	64	16
M50 pskA-*mgrB*-WT[c]	≤0.25	0.25
M50 pskA-*mgrB*-mutant[c]	64	64

301

302 [a]To prevent plasmid loss, MICs of completed strains were performed in presence of apramycin 50 mg/L.

303 [b]Experiments were performed in biological triplicates.

304 [c]*mgrB* from A58243 was used as wild-type (WT) while *mgrB* from M50 was used as *mgrB*-mutant.

Figure 1. (A) Relative transcriptional levels of TCS evaluated in polymyxin-resistant *K.* variicola isolates. (B) Multiple nucleotide alignment of *mgrB* variants detected in this study. **(C)** Multiple protein alignment of MgrB proteins. Red lines highlight the change in nucleotide/aminoacid sequences.